EXHIBIT 6

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Expression Characteristics of Two Potential T Cell Mediator Genes

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T lymphocyte subset-specific cDNA clones were recently isolated by a modified differential screening procedure. The expression patterns of two of these cDNAs, designated as 4-1BB and L2G25B, were studied in greater detail. Nucleotide sequence comparison revealed that 4-1BB was not previously recognized. Although the L2G25B sequence had been recognized recently, the function of the encoded molecule has yet to be well studied. The transcripts of the two cDNAs were inducible by concanavalin A in mouse spleen cells, cloned helper T cells (L2), cloned cytolytic T cells (L3), and cytolytic T cell hybridomas. They were also inducible with stimulation through antigen receptor (TCR), with immobilized anti-TCR antibody in cloned T cells L2, dB45, and L3. Concanavalin A inducibility was inhibited by cyclosporin A. They were not inducible by IL-2 stimulation. The expression patterns of these transcripts were similar to those of IFN-γ, except that the level of transcripts of the two cDNAs was at least fivefold lower than that of IFN-γ, and the peak level of expression occurred earlier. These data suggest that L2G25B and 4-1BB may represent new T cell mediators. © 1989 Academic Press, Inc.

INTRODUCTION

T lymphocytes play a central role in the immune network both as effectors and regulators. They are composed of subsets endowed with distinct helper, suppressor, and cytolytic capabilities. These functions may be mediated by surface receptors and subset-specific immune effectors which are elaborated and secreted after stimulation either with lectin or specific antigen or immobilized monoclonal antibody (mAb) against T cell antigen receptor (TCR) (1–4). The genes for a number of the subset-specific T cell effector molecules have been cloned, but not all activities are correlated with the cloned genes. Identification and demonstration of such unrecognized molecules can uncover hitherto-unknown functions of T cells.

This laboratory has recently cloned a series of T cell subset-specific cDNAs from cloned helper T (HTL) L2 and cloned cytolytic T (CTL) L3 cells by employing a modified differential screening procedure (5). Nucleotide sequences of two cDNA clones, L2G25B and 4-1BB, were determined (6). The deduced amino acid sequences revealed that both contain putative leader sequences. The protein encoded by 4-1BB had a potential membrane anchor segment and other features also seen in known receptor proteins (6). A human homolog of L2G25B was reported (7) and the same

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mouse sequence has been isc
characteristics of expression c
L2 and L3 are the focus of the
of inducibility and expression.

MAT

Cells. Cloned murine CTL
1^+, L3T4$^-$, and H-2L^d reactive
1^+, Lyt-2$^-$, L3T4$^+$, and Mls$^{a/d}$
PN37 are derived by the fusic
(11). They are Thy-1^+, LFA-1^+
L2 cells were stimulated with
lized anti-TCR mAb F23.1 (1
cells were stimulated with con
clonotypic anti-TCR mAb 384
human IL-2 (100 μg/ml) (Cetus
of 2.5×10^6/ml. In other exp
alone, concanavalin A plus cyc
mycin D (1 μg/ml) for 6 hr. L2
by David Lancki at the Univers
Md90, PN37, BW5147, and
lin A (5 μg/ml) at a cell conce
Md90 and PN37 cells was mo
production. Mouse thymoma
noylphorbol-13-acetate (TPA,
20 hr. Stimulation was monitor
C57BL/6, BALB/c, or Swiss W
navalin A (5 μg/ml) at a cell con
K46 (17), rat NK cell LGL (18
were not stimulated with any o
Isolation of T cell-specific cL
cDNAs that are specific for T ce
negative differential screening a
T cell-specific cDNAs were furt
for cloned HTL L2 or cloned C
nucleotide and deduced amino a
L2G25B, were reported elsewh
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rophage cell line RAW264.7 (8).
RNA blot hybridization. Tota
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England Nuclear, Boston, MA).
translation and used as probes.
50% formamide, 5× SSC (1× S
0.1% SDS, 250 μg/ml of salmon
washed at room temperature for
min in 0.1× SSC and 0.1% SDS

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mouse sequence has been isolated from the murine macrophage cell line (8). The characteristics of expression of the two cDNA clones which were expressed in both L2 and L3 are the focus of the present report. These clones, because of their patterns of inducibility and expression, may represent potential new T cell mediators.

MATERIALS AND METHODS

Cells. Cloned murine CTL L3 (9) and dB45 cells (10) are Thy-1,2$^+$, Lyt-2$^+$, LFA-1$^+$, L3T4$^-$, and H-2L^d reactive. Cloned murine HTL L2 cells (9) are Thy-1,2$^+$, LFA-1$^+$, Lyt-2$^-$, L3T4$^+$, and Mls$^{a/d}$ reactive. The cytolytic T cell hybridomas Md90 and PN37 are derived by the fusion of BW5147 thymoma and BALB/c antiEL-4 CTL (11). They are Thy-1$^+$, LFA-1$^+$, Lyt-2$^-$, L3T4$^-$, and H-2D^b reactive.

L2 cells were stimulated with concanavalin A (10 μg/ml) for 14 hr, or with immobilized anti-TCR mAb F23.1 (12) for 6 hr at a cell concentration of 10^6–10^7/ml. L3 cells were stimulated with concanavalin A (2 μg/ml) for 14 hr, or with immobilized clonotypic anti-TCR mAb 384.5 (13) for various time periods, or with recombinant human IL-2 (100 μg/ml) (Cetus Corp., Emeryville, CA) for 6 hr at a cell concentration of 2.5×10^6/ml. In other experiments, L3 cells were treated with concanavalin A alone, concanavalin A plus cyclosporin A (0.2 μg/ml), or concanavalin A plus actinomycin D (1 μg/ml) for 6 hr. L2, L3, and dB45 cell preparations were kindly provided by David Lancki at the University of Chicago, Illinois.

Md90, PN37, BW5147, and CTLLA11 (14) cells were stimulated with concanavalin A (5 μg/ml) at a cell concentration of 5×10^6 cells/ml for 4 hr. Stimulation of Md90 and PN37 cells was monitored by increased cytotoxicity and increased IL-2 production. Mouse thymoma EL-4 cells (15) were stimulated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA, 10 ng/ml) at a cell concentration of 1.0×10^6/ml for 20 hr. Stimulation was monitored by IL-2 assay (16). Splenocytes were obtained from C57BL/6, BALB/c, or Swiss Webster mouse spleens and were stimulated with concanavalin A (5 μg/ml) at a cell concentration of 5.0×10^6/ml for 14 hr. B cell lymphoma K46 (17), rat NK cell LGL (18), and mouse melanoma, Cloudman S-91 (19) cells were not stimulated with any of the above reagents.

Isolation of T cell-specific cDNA clones. We have previously isolated a group of cDNAs that are specific for T cells in contrast to B cells, employing both positive and negative differential screening and RNA blot analysis of various lymphoid cells. The T cell-specific cDNAs were further studied to determine whether they were specific for cloned HTL L2 or cloned CTL L3. The identity of these cDNA sequences, and nucleotide and deduced amino acid sequences of previously unrecognized 4-1BB and L2G25B, were reported elsewhere (6). We learned, however, that the L2G25B sequence was isolated recently from lipopolysaccharide (LPS)-stimulated murine macrophage cell line RAW264.7 (8).

RNA blot hybridization. Total cytoplasmic RNA or poly(A)$^+$ RNA was fractionated on 1.2% agarose–formaldehyde gels and transferred to Gene Screen Plus (New England Nuclear, Boston, MA). Gel-purified cDNA inserts were ^{32}P-labeled by nick translation and used as probes. Filters were prehybridized and hybridized at 42°C in 50% formamide, 5× SSC (1× SSC–150 m*M* NaCl, 15 m*M* sodium citrate, pH 7.0), 0.1% SDS, 250 μg/ml of salmon sperm DNA, and 10% dextran sulfate. Filters were washed at room temperature for 15 min in 2× SSC and 0.1% SDS, and at 42°C for 5 min in 0.1× SSC and 0.1% SDS several times. When a Northern blot of Gene Screen

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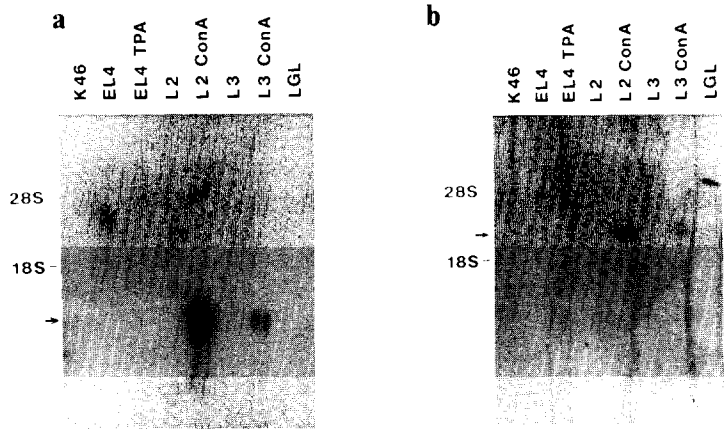

FIG. 1. T cell-specific expression of L2G25B and 4-1BB mRNA. Poly(A)$^+$ mRNA was prepared from mouse B cell line (K46), unstimulated EL-4 (EL-4), TPA-stimulated EL-4 (EL-4 TPA), and rat NK cell line (LGL); and total RNA was prepared from unstimulated L2 (L2), concanavalin A-stimulated L2 (L2 Con A), unstimulated L3 (L3), and concanavalin A-stimulated L3 (L3 Con A). Ten micrograms of total RNA or ten micrograms of poly(A)$^+$ RNA was fractionated on a formaldehyde/agarose gel, transferred to Gene Screen Plus, and hybridized to ^{32}P-labeled L2G25B (a) and 4-1BB (b) sequentially. Positions of 28 and 18 S rRNA markers are indicated. An arrow indicates the specific hybridization signal.

Plus was used multiple times for hybridization, the previous probe was removed by treating the membrane in 10 m*M* Tris–HCl (pH 7.0), 0.2% SDS at 85° for 1 hr.

RESULTS

L2G25B and 4-1BB Are Expressed Preferentially in T Cells

L2G25B was isolated from an L2 cDNA library, and 4-1BB was isolated from an L3 cDNA library by a modified differential screening (5). As shown in Figs. 1a and 1b, L2G25B and 4-1BB were expressed preferentially in L2 and L3 cells only after concanavalin A stimulation. The sizes of transcripts were approximately 800 bases for L2G25B and 2400 bases for 4-1BB. The abundance of the two transcripts was 5- to approx 10-fold higher in L2 cells than in L3 cells. The two transcripts were not detectable in K46 B cells, EL-4 thymoma cells, or rat large granular lymphocytes. L2G25B mRNA was consistently more abundant than 4-1BB mRNA.

L2G25B and 4-1BB mRNA Are Inducible by TCR Stimulation, but Not by IL-2 Stimulation

The inducibility of the two cDNA clones was tested after L3 TCR stimulation by clonotypic anti-TCR mAb, 384.5, or IL-2. As shown in Figs. 2a and 2b, the expression of the two cDNAs was inducible by TCR stimulation, but not by IL-2 stimulation in L3 cells. L2G25B mRNA was detectable at 0.5 hr after TCR stimulation, peaked at 6 hr, and decreased thereafter until at least 24 hr. 4-1BB mRNA was detectable at a very low level in unstimulated L3 cells in this experiment. The induction of 4-1BB mRNA occurred approximately 6 hr after TCR stimulation and remained level until 24 hr.

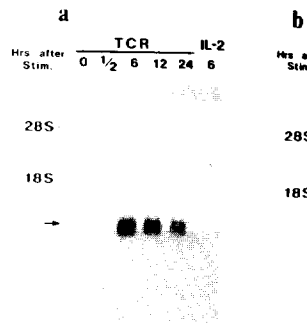

FIG. 2. Patterns of L2G25B and
cells were stimulated with clonotyp
Ten micrograms of total RNA was
Plus, and hybridized to ^{32}P-labeled
is a serine protease cDNA isolated f
is used to show that each lane cont
markers are indicated. An arrow in

Figure 2c shows the kineti
used in Fig. 2a or 2b. IFN-γ
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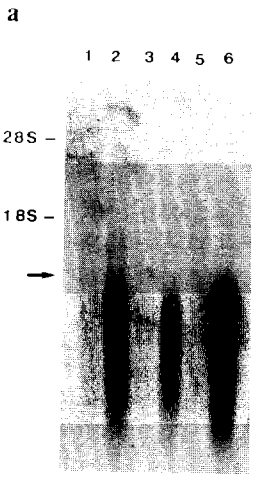

FIG. 3. Expression of L2G25B and
dB45 cells were stimulated with anti-
mAb 384.5 for 6 hr. Ten micrograms
2), unstimulated dB45 (lane 3), stim
(lane 6) was fractionated on formalde
hybridized to ^{32}P-labeled L2G25B (a)
was degraded and detected as RNA in

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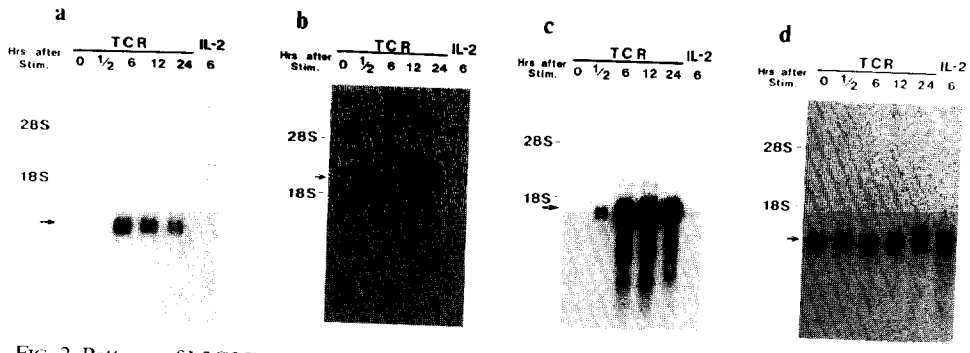

FIG. 2. Patterns of L2G25B and 4-1BB mRNA expression after TCR stimulation or IL-2 treatment. L3 cells were stimulated with clonotypic anti-TCR mAb 384.5 for 0, ½, 6, 12, or 24 hr, or with rIL-2 for 6 hr. Ten micrograms of total RNA was fractionated on a formaldehyde/agarose gel, transferred to Gene Screen Plus, and hybridized to ^{32}P-labeled L2G25B (a), 4-1BB (b), IFN-γ (c), and L3G10#6 (d) cDNA. L3G10#6 is a serine protease cDNA isolated from L3 cell cDNA library, which is identical to HF gene (29). L3G10#6 is used to show that each lane contains an almost equal amount of RNA. Positions of 28 and 18 S rRNA markers are indicated. An arrow indicates the specific hybridization signal.

Figure 2c shows the kinetics of IFN-γ mRNA expression in the same RNA blot as used in Fig. 2a or 2b. IFN-γ mRNA was detectable at 0.5 hr after TCR stimulation, peaked at 12 hr, and declined slightly until 24 hr. There was a low level of IFN-γ mRNA in unstimulated L3 cells. When we compared the peak levels of L2G25B and 4-1BB mRNA with that of IFN-γ mRNA, IFN-γ mRNA was at least 5-fold higher than that of L2G25B mRNA and at least 25-fold higher than that of 4-1BB mRNA.

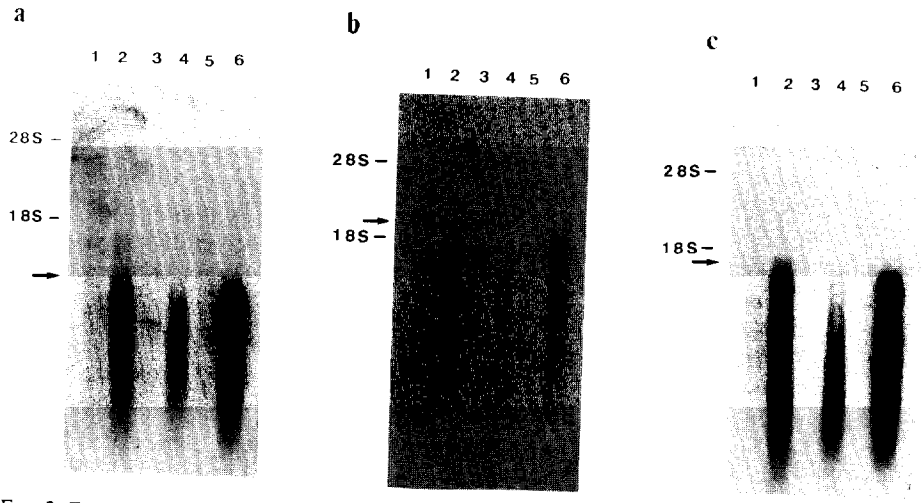

FIG. 3. Expression of L2G25B and 4-1BB mRNA in HTL L2 and CTL dB45 cells. HTL L2 and CTL dB45 cells were stimulated with anti-TCR mAb F23.1 for 6 hr. L3 cells were stimulated with anti-TCR mAb 384.5 for 6 hr. Ten micrograms of total RNA from unstimulated L3 (lane 1) and stimulated L3 (lane 2), unstimulated dB45 (lane 3), stimulated dB45 (lane 4), unstimulated L2 (lane 5), and stimulated L2 (lane 6) was fractionated on formaldehyde/agarose denaturing gel, transferred to Gene Screen Plus, and hybridized to ^{32}P-labeled L2G25B (a), 4-1BB (b), and IFN-γ (c) cDNA. A fraction of RNA in each lane was degraded and detected as RNA in lower molecular sizes.

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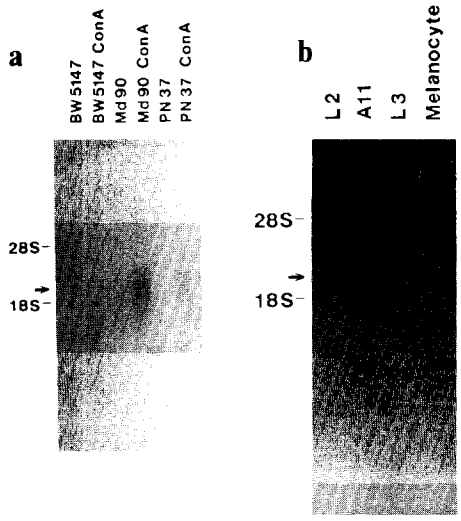

FIG. 4. Expression of 4-1BB mRNA in concanavalin A-stimulated hybridomas PN37 and Md90, and in a CTL CTLLA11. (a) Ten micrograms of poly(A)$^+$ mRNA from BW5147, PN37, and Md90 cells, both stimulated and unstimulated, was fractionated, transferred to nitrocellulose filter, and probed with ^{32}P-labeled 4-1BB cDNA probe. (b) Ten micrograms of poly(A)$^+$ mRNA from mouse melanoma cells (melanocyte) and 10 μg of total RNA from unstimulated L2 (L2), L3 (L3), and stimulated CTLLA11 (A11) cells were fractionated, transferred to Gene Screen Plus, and hybridized to ^{32}P-labeled 4-1BB cDNA probe.

Figure 2d demonstrates that all six lanes contained almost identical amounts of RNA. The probe was a serine protease cDNA (L3G10#6) isolated from L3 cells (5). In summary, the pattern of the two cDNA expressions was similar to that of IFN-γ expression upon TCR stimulation.

L2G25B and 4-1BB mRNA Are Inducible by TCR Stimulation in Other Cloned HTL, CTL, and Hybridomas

As shown in Figs. 3a and 3b, L2G25B and 4-1BB mRNA are also inducible in HTL L2 and CTL dB45 after TCR stimulation with anti-TCR mAb F23.1. The mRNA level for the two cDNAs was also much lower than that of IFN-γ in L2 and dB45 cells (Fig. 3c). L2 cells show the highest level of expression of the three cell clones. We also found that 4-1BB mRNA was inducible by concanavalin A in two cytotoxic hybridomas, PN37 and Md90 (Fig. 4a), and a CTL line CTLLA11 (Fig. 4b).

Effects of Cyclosporin A on L2G25B and 4-1BB Transcription

We next examined the effect of cyclosporin A on RNA expression of L2G25B and 4-1BB. Cyclosporin A inhibits mitogen- or antigen-induced T cell proliferation (20–22). It has also been shown to block the induction of expression of several lymphokine genes, including IL-2 and IFN-γ (23–25). The inhibition of lymphokine production occurs at a pretranslational level (24–26). In contrast, cyclosporin A appears to have no effect on the inducible expression of c-*fos* and IL-2 receptor genes in T cells (23). As shown in Figs. 5a and 5b, cyclosporin A inhibited the induced accumulation of L2G25B and 4-1BB mRNA. The same findings were seen with IFN-γ (Fig. 5c). Fig-

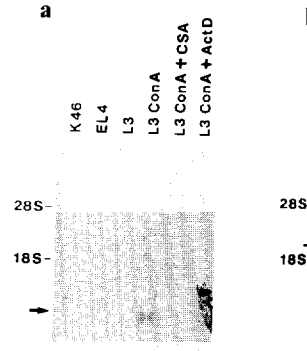

FIG. 5. Effect of cyclosporin A
with concanavalin A, concanavali
micrograms of total RNA from uns
navalin A plus cyclosporin A-treat
treated L3 (L3 Con A + ActD) cell
EL-4 cells (EL-4) were fractionated
labeled L2G25B (a), 4-1BB (b), IFN
the level of L3G10#6 mRNA, but a
mRNA species. An arrow indicates

ure 5d shows that cyclospor
protease (probe L3G10#6) m
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L2G25B and 4-1BB mRNA A

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able in concanavalin A-stimul
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and 6b. These data suggest th
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are as follows: (1) The mRNA
The mRNAs of the two genes
induced by concanavalin A or

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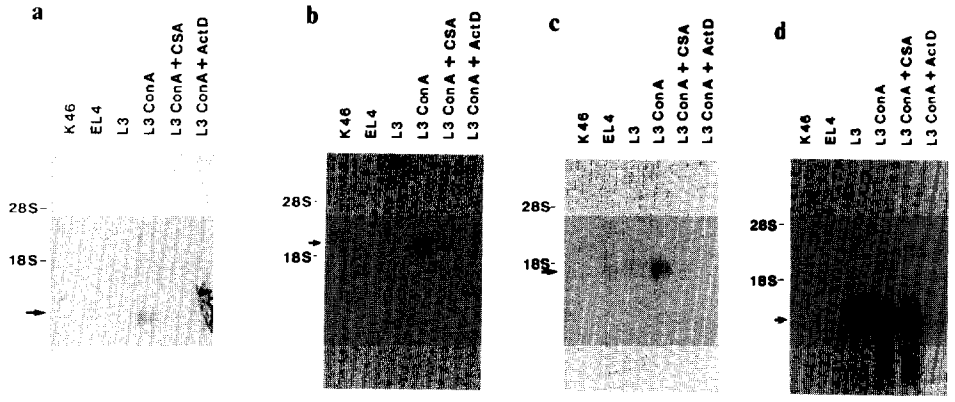

FIG. 5. Effect of cyclosporin A on L2G25B and 4-1BB mRNA expression. L3 cells were stimulated with concanavalin A, concanavalin A plus cyclosporin A, or concanavalin A plus actinomycin D. Ten micrograms of total RNA from unstimulated L3 (L3), concanavalin A-stimulated L3 (L3 Con A), concanavalin A plus cyclosporin A-treated L3 (L3 Con A + CsA), and concanavalin A plus actinomycin D-treated L3 (L3 Con A + ActD) cells and 10 μg of poly(A)$^+$ mRNA from K46 (K46) and TPA-stimulated EL-4 cells (EL-4) were fractionated, transferred to Gene Screen Plus membrane, and hybridized to ^{32}P-labeled L2G25B (a), 4-1BB (b), IFN-γ (c), and L3G10#6 (d) cDNA. Cyclosporin A treatment did not alter the level of L3G10#6 mRNA, but almost completely abrogated the induced expression of the other three mRNA species. An arrow indicates a specific hybridization signal.

ure 5d shows that cyclosporin A had minimal or no effect on the level of a serine protease (probe L3G10#6) mRNA, and shows that the three lanes contained almost equal amounts of RNA (EL-4 or K46 cells did not express L3G10#6 mRNA). These data strongly suggest that L2G25B and 4-1BB expression may show some of the same activation requirements as other known lymphokines.

L2G25B and 4-1BB mRNA Are Inducible in Normal Mouse Spleen Cells

To find out whether or not the expression of these genes was unique to certain cloned T cells or hybridoma cells, splenocytes from C57BL/6 and BALB/c mice were stimulated with concanavalin A and tested for mRNA expression. As shown in Figs. 6a and 6b, the two mRNAs were detectable after concanavalin A stimulation in C57BL/6 and BALB/c mouse splenocytes. They were also inducible in Swiss Webster mouse splenocytes (data not shown). As shown in Fig. 6c, IFN-γ mRNA was detectable in concanavalin A-stimulated BALB/c splenocytes (for unknown reasons, INF-γ mRNA was not detectable in concanavalin A-stimulated C57BL/6 splenocytes in this experiment). RNA preparations for Fig. 6c were different from those for Figs. 6a and 6b. These data suggest that these molecules may be induced in normal mouse spleen cells by appropriate stimuli, as in the cloned T cells.

DISCUSSION

L2G25B and 4-1BB share properties of soluble T cell mediators. The properties are as follows: (1) The mRNAs of the two are preferentially expressed in T cells. (2) The mRNAs of the two genes are present in undetectable amounts in T cells until induced by concanavalin A or by TCR stimulation. (3) The patterns of expression are

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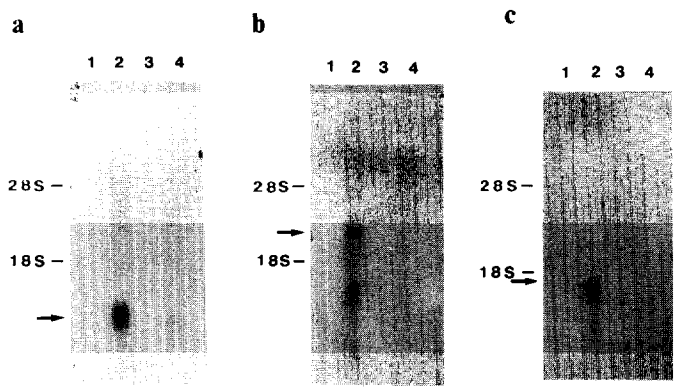

FIG. 6. Expression of L2G25B and 4-1BB mRNA in mouse splenocytes. Splenocytes were obtained from C57BL/6 and BALB/c mice and stimulated with concanavalin A for 14 hr. Ten micrograms of total RNA from unstimulated BALB/c (lane 1) and stimulated BALB/c (lane 2), unstimulated C57BL/6 (lane 3), and stimulated C57BL/6 (lane 4) splenocytes was fractionated, transferred to Gene Screen Plus, and hybridized to ^{32}P-labeled L2G25B (a), 4-1BB (b), and IFN-γ (c) cDNA.

very similar to that of the lymphokine IFN-γ. (4) Cyclosporin A inhibits the induced mRNA expression corresponding to the two cDNAs. In addition, sequence analysis of L2G25B showed features consistent with several analyzed lymphokine cDNAs; for example, the small size of mRNA, potential signal sequence, and repeated AUUUA element in 3′ untranslated region (27). In contrast, while the 4-1BB exhibits expression patterns which resemble those of the lymphokine mRNAs, the sequence analysis of this cDNA appears consistent with those of known receptor proteins. It would be interesting, therefore, to determine the function of 4-1BB. Since Davatelis *et al.* (8) isolated the cDNA clone identical to L2G25B from the LPS-stimulated RAW264.7 cell line, we tested the mRNA expression of L2G25B and 4-1BB in the RAW264.7 cells. L2G25B mRNA was inducible within 30 min by LPS stimulation in the cells, but 4-1BB mRNA was not detectable over a 36-hr induction period. Interestingly, the induction of L2G25B mRNA was not inhibited by cyclosporin A in the RAW264.7 macrophage line (Fig. 7).

Using the same concanavalin A-stimulated L2 cells, Prystowsky *et al.* (28) identified 10 different lymphokine activities from culture supernatants. They include IL-2, IL-3, B cell stimulatory factor, granulocyte/macrophage colony-stimulating factor, IFN-γ, and five unidentified factors which affect macrophage activities. In the course of the studies, we isolated and identified cDNAs for IL-2, IL-3, GM-CSF, T cell replacing factor, and proenkephalins from our concanavalin A-stimulated L2 cDNA library (5, 6). We suggest, therefore, that L2G25B might represent the novel soluble mediators of Prystowsky *et al.* (28), which affect macrophage activities. However, the nature of the 4-1BB gene product is difficult to predict.

By applying a modified differential screening of L2 and L3 cDNA library, two novel T cell genes were isolated. The two T cell genes were expressed at low levels compared with IFN-γ expression level, and these genes shared expression properties with several of the known lymphokines. Correlation of the T cell molecules with functional activities is the next critical step.

FIG. 7. Expression of L2G25B m
LPS (*Escherichia coli* 0127:B8, Sig
contains 20 μg of total RNA from
(lane 2), stimulated with LPS + cy
stimulated with LPS + cyclosporin
agarose gel, transferred to Gene Scr
tions of 28 and 18 S mRNA mark
signal.

The author thanks Dr. Sherman
David Lancki, and Mike Prystowsk
LGL; Dr. John Farrar for EL-4 and
Ding-E Young for CTLLA11 cells;
Cloudman S-91 cells. I also thank D
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Wall, and Mrs. Mary Kiefer for typi
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1. Kronenberg, M., Siu, G., Hood,
2. Smith, K., *Annu. Rev. Immunol*
3. Kishimoto, T., *Annu. Rev. Imm*
4. Moldwin, R., Lancki, D., Harold
5. Kwon, B., Kim, G., Prystowsky
Acad. Sci. USA **84**, 2896, 198
6. Kwon, B., and Weissman, S., *Pr*

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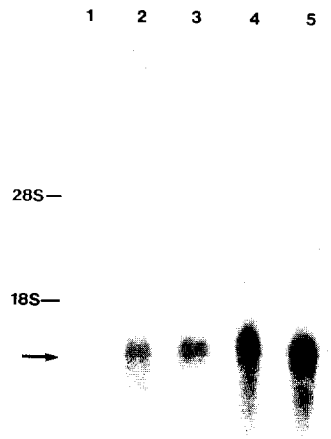

FIG. 7. Expression of L2G25B mRNA in RAW264.7. RAW264.7 cells were stimulated with 1 μg/ml of LPS (*Escherichia coli* 0127:B8, Sigma, St. Louis, MO), or LPS + cyclosporin A (0.2 μg/ml). Each lane contains 20 μg of total RNA from RAW264.7 cells, unstimulated (lane 1) or stimulated with LPS for 2 hr (lane 2), stimulated with LPS + cyclosporin A for 2 hr (lane 3), stimulated with LPS for 6 hr (lane 4), or stimulated with LPS + cyclosporin A for 6 hr (lane 5). The RNA was fractionated on a formaldehyde/agarose gel, transferred to Gene Screen Plus membrane, and hybridized to ^{32}P-labeled L2G25B. The positions of 28 and 18 S mRNA markers are each indicated. An arrow indicates the specific hybridization signal.

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Thymus-independent prim
untreated and TLI-treated NZ
a low primary response to *Br*
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mary antibody response at da
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or Ficoll were masked by high
in the anti-BA response, spon
markedly decreased after TLI

NZB/NZW F1 female mic
to human systemic lupus er
antibodies, hypergammaglo
tion, immune complex glom
tures make these F1 mice a
various immunologic abnorm
ble to human disease.

It has been reported, as the
ous IgG secretion by spleen c
antibodies (2, 3), as well as a
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purified protein derivative of
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Total lymphoid irradiation
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with moderate or advanced r
month after TLI, there is an e

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